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Mitigating AFB1 and OTA hepatic residues using pumpkin and milk fermented whey as functional ingredients

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ABSTRACT

The consumption of antioxidant-rich commodities acts as a preventive measure to regulate mycotoxin levels, which pose a significant threat to human and animal health worldwide. This study aimed to evaluate the potential reduction of mycotoxin liver bioaccumulation through an *in vivo* experimental study on 120 rats fed with pumpkin, milk fermented whey, or combinations. The levels of Aflatoxin B1 (AFB1) and Ochratoxin A (OTA) in liver were monitored through Ultra High-Resolution Liquid Chromatography-Orbitrap Mass Spectrometry (UHPLC-Q-Orbitrap HRMS). The methodology was appropriately optimized according to current regulations. Limit of quantification (LOQ) for AFB1 and OTA were 0.78 and 6.25 ng/g respectively, whereas recovery rates were up to 70% for both mycotoxins. Mycotoxin residues in the liver of rats fed with functional ingredients showed up to a 100 % reduction for AFB1 and 80% for OTA. The combination of pumpkin and fermented whey in feed formulations has proven to be an effective approach easily implemented by the food industry to safeguard consumer health.

1. Introduction

Currently, mycotoxin contamination is a major concern for human and animal health worldwide. The principal underlying cause derives from the presence of several species of filamentous fungi, which are capable to naturally secrete these secondary metabolites at different points of the food chain (Manyes & Font, 2023). Last year, the European Union has updated the legislation on mycotoxins in order to include ergot alkaloids and decrease maximum levels for several mycotoxins in different food matrices and feed to ensure they are not harmful to human or animal health (European Commission, 2023).

Currently, the most toxic have proven to be Aflatoxin B1 (AFB1) and Ochratoxin A (OTA), classified as carcinogenic (group 1) and possibly carcinogenic for humans (group 2B), respectively (IARC, 2011). According to the literature, AFB1 is strongly implicated in the onset of liver cancer, as the liver is its primary site of metabolism. More specifically, it is able to produce the formation of reactive conjugates with DNA, producing the disruption of transcription processes and consequent aberrant cell proliferation (Theumer et al., 2003). Similarly, it has been observed that OTA is swiftly absorbed after oral ingestion in different animal species and then accumulated primarily in liver and kidney (Tao et al., 2018). Moreover, OTA has proven to have long persistence in the organism and, therefore, the steady exposure could interact with other mycotoxins that co-contaminate foodstuffs (EFSA, 2010). The combined exposure to AFB1 and OTA, for instance, could exacerbate its toxicological effect or, on the contrary, further produce an antagonistic effect on its toxicokinetic. On the one hand, a few toxicological *in vivo* studies reported conflicting results about this co-occurrence and the alteration of animals' physiological parameters such as mortality, body and organ weights. On the other hand, in certain cases OTA was able to inhibit AFB1 damages (Corcuera et al., 2012).

Despite their severe toxicity and bioaccumulation, the development of analytical methodologies to simultaneously quantify AFB1 and OTA residues in biological tissues, especially liver and kidney, are currently limited. The complexity of their application mainly arises from the limited sample volume, the number of laboratory animals, and the

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[1] This paper is dedicated to the memory of our dear Professor Alberto Ritieni who passed away last 13 June 2023.

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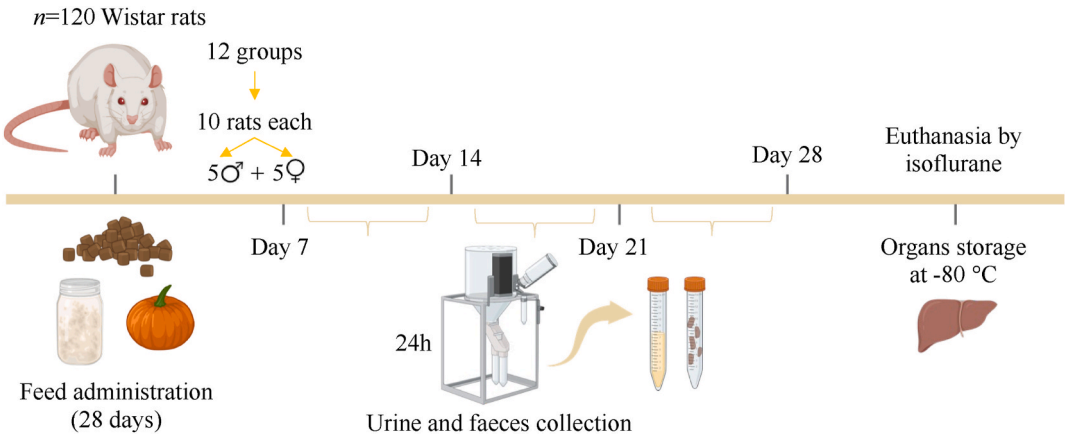

Fig. 1. Graphical representation of the *in vivo* experimental design.

specific nature of the tissue (Herzallah, 2009; Arce-López et al., 2020). Even though, in recent years the use of liquid chromatography coupled with mass spectrometry (LC-MS/MS) has become a primary technique in a wide variety of samples, including animal matrices and biological fluids (Solfrizzo et al., 2011; Corcuera et al., 2011; Cao et al., 2018; Galindo et al., 2024).

Conversely, although mycotoxins presence in food commodities is significant, diverse substances are able inhibit the absorption by impeding their access in the bloodstream from the gut. Among them, fermented foods have shown a remarkable success in reducing mycotoxins contamination and at the same time, they constitute an improvement in products' quality from the consumer point of view (Agriopoulou et al., 2020). Strategies based on the use of lactic acid bacteria (LAB), for instance, are currently considered an optimal approach being safe for human and maintaining the nutritional value of food (Badji et al., 2023). Likewise, the use of commodities rich in antioxidants results in a preventive mode to control mycotoxins levels. They neutralize reactive oxygen species, thereby protecting against potential mycotoxin damage. (Del Fabbro et al., 2024).

Deepening the use of functional ingredient as a mitigation strategy, milk fermented whey (FW) and pumpkin (P), rich in antioxidant and bioactive substances, represent a good ally for reducing mycotoxins bioaccessibility and bioavailability (Escrivá et al., 2021; Cimbalo et al., 2023). In fact, recent investigations highlighted the protective role of FW and pumpkin P mixture on AFB1 and OTA-induced alterations at hepatic, renal, neuronal, immune and intestinal level (Frangiamone et al., 2024). The whey from goat milk is a common by-product in cheese production at dairy industry. The fermentation using LAB enhances the nutritional value of the whey to be used as functional ingredient in order to promote circular economy in dairy industry.

Therefore, the present study aimed to (i) optimize a reliable analytical methodology through Ultra High-Resolution Liquid Chromatography-Orbitrap Mass Spectrometry (UHPLC Q-Orbitrap HRMS) to detect AFB1 and OTA residues in rat liver and (ii) concurrently determine the potential protective role of FW and P to diminish mycotoxins accumulation in rat liver through an *in vivo* experimental study on 120 rats fed with pumpkin, milk fermented whey, or combinations.

2. Material and methods

2.1. Chemicals and reagents

Methanol (MeOH), acetonitrile (ACN), formic acid (FA), glacial acetic acid (AA) and H_2O of analytical grade for chromatography were acquired from Merck (Milan, Italy). Ammonium formate, sodium chloride (NaCl), anhydrous magnesium sulfate ($MgSO_4$) and mycotoxin standards (purity ≥98%) were purchased from Sigma-Aldrich (Milan, Italy). For preparing stock solution of analytes, 1 mg of each mycotoxin was diluted in 1 mL of methanol. From these, a working solution which included AFB1 and OTA was diluted at the concentrations required for the spiking experiments (5, 25, and 50 μg/kg) and calibration curve (400 μg/kg). All the solutions were kept under safe conditions at −20 °C for a maximum of 1 month.

2.2. In vivo experimental design

One hundred and twenty specimens of male and female Wistar rats (260–340 g) of 8 weeks-old were acquired from Envigo RMS Spain S.L. (Barcelona, Spain). Animals were housed in polycarbonate cages in a windowless room with a 12h light-dark cycle under specific conditions of temperature (22 °C) and relative humidity (45–65%) at the Burjassot campus facilities managed by the Central Service for Experimental Research (Universitat de València, Spain). During the procedures, nitrile gloves and FFP3 masks were employed to maintain sterility conditions such as manipulation of exposed animals or contaminated samples. This project was approved by the institution Animal Care and Use Committee of the University of Valencia (2021/VSC/PEA/0112).

For the experimental procedures, rats were randomly distributed into twelve groups (10 rats for each, 5 males and 5 females), as described in Table S1. After 7 days of acclimatization, the control group (n=1) was fed with normal uncontaminated feed whereas the test groups (n=11) were supplied with mycotoxins and FW or pumpkin-containing feed for 28 days. Feed preparation was carried out as described by Vila-Donat et al., 2024. FW and P were used as functional ingredients, either individually or in combination, to replicate a realistic scenario of the Mediterranean diet. Furthermore, consuming a single functional compound is unlikely, as natural foods typically contain a variety of bioactive compounds. For the feeds containing functional ingredients, 35 g of FW and P were added to each during the preparation, corresponding to 1% (w/w). The doses were chosen based on a bioaccessibility study previously conducted by Escrivá et al. (2021), which used bread enriched with FW and P to reduce the levels of AFB1 and OTA following the intestinal digestion process. Mycotoxin concentrations in feed result from a natural contamination of flours performed to simulate a realistic scenario of daily ingestion, by comparing Mediterranean diet and humans' habits (WHO, 2018). AFB1 and OTA levels were detected in the 12 different groups by using a HPLC-FLD method (μg/g) (Table S1, Vila-Donat et al., 2024). In general, the development of OTA in feed was higher (5.44–8.83 μg/g) than the AFB1 one (4.3–5.2 μg/g). However, the highest concentrations of toxins mixture were reported in presence of both functional ingredients (5.2±0.9 μg/g AFB1; 8.8±0.4 μg/g OTA). After each week of exposure, the experimental rats were placed into metabolic cages once per week during 24 h in order to calculate

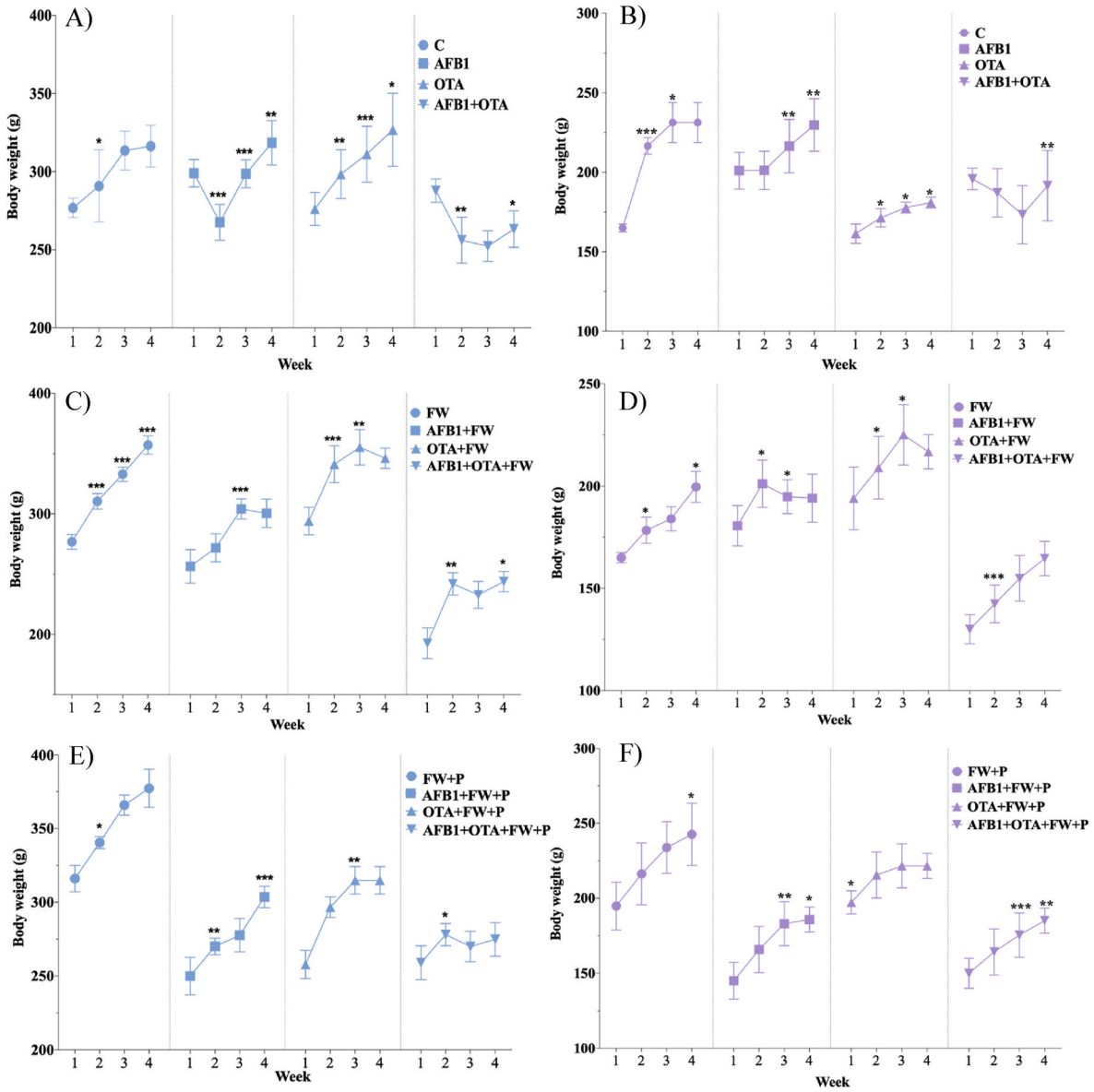

Fig. 2. Changes in the body weight (g) of male (A) and female (B) rats fed uncontaminated feed (control), AFB1, OTA and AFB1+OTA; Male (C) and female (D) rats fed FW enriched feed, AFB1+FW, OTA + FW, AFB1+OTA + FW; Male (E) and female (F) rats fed FW+P enriched feed, AFB1+FW+P, OTA+FW+P, AFB1+OTA+FW+P; Body weights of rats were measured every 7 days after feed administration, three replicates of each measurement were made. Data were expressed as mean ± standard deviation (SD). Symbols represent week 1, 2, 3 and 4. *p<0.05; **p<0.01; ***p<0.001. FW: Fermented whey, P: Pumpkin, AFB1: Aflatoxin B1, OTA: Ochratoxin A.

cumulative daily feed intakes and toxins ingestion. After 28 days of exposure to simulate a sub-chronic study, the rats received an anesthesia overdose, administered as 1.72% isoflurane, to ensure they did not regain consciousness at any stage, particularly during dissection. Afterwards, liver was removed and stored at −80 °C. The experimental procedures of the *in vivo* study are illustrated in Fig. 1.

2.2.1. *Body weights, feed intake and mycotoxin daily intake*

Cumulative weekly feed intakes (g) were determined by calculating the difference of food given and food left over, each week. Body weights (BW) were also determined at the same time. In addition, the probable daily intake (PDI) was calculated considering rats BW, average daily intake of feed during three weeks and toxins concentrations in the feed. The PDI of each mycotoxin was assessed with the following equation (Abrunhosa et al., 2016):

$$PDI_m = (C_{m\ x}\ K) / BW \quad (1)$$

where PDIm is the probable daily intake (μg kg^{-1} BW d^{-1}) for each mycotoxin; Cm is the mean content of the mycotoxin (μg kg^{-1}); K is the average consumption of the commodity (μg d^{-1}) and BW is the body weight of each population group.

2.3. *Mycotoxin extraction*

Mycotoxins extraction was initiated from 0.2 g of frozen liver grounded in ultra-pure H_2O (analytical grade) with an Ultra-turrax Digital High-Speed Homogenizer (IKA®, Staufen, Germany). Samples were then mixed with 5 mL of ACN acidified with 0.1% of acetic acid, vortexed for 1 min and sonicated for 5 min. The mixture was subjected to solid-phase extraction by adding 0.2 g of $MgSO_4$ and 0.05 g of NaCl, then vortexed for 30 s. Afterwards, they were centrifuged at 3000 rpm for 5 min at 4 °C (Sigma Centrifuge 3K15; Sigma, Germany) and supernatant was collected into new centrifuge tubes. The final extract was

evaporated to dryness under nitrogen flow and resuspended in mobile phase. Lastly, it was filtered through a nylon filter (0.22 μm), opportunely diluted until analysis using UHPLC-Q-Orbitrap HRMS.

2.4. UHPLC-Q-Orbitrap HRMS analysis

The UHPLC-Q-Orbitrap HRMS methodology employed was previously optimized by Lima da Silva et al. (2023) for mycotoxin identification with slight modifications. A UHPLC system (Dionex UltiMate 3000, Thermo Fisher Scientific, Waltham, MA, USA) consisting of a solvent delivery pump (LPG-3400RS), a micro-degasser system (GPL-3400RS), a refrigerated autosampler (WPS-3000RS), and a thermostatic column oven (TCC-3000SD) was employed for chromatographic analysis. Compound separation was carried out using a Kinetex 2.6 μm column (100 × 2.1 mm, Phenomenex, Torrance, CA, USA) coupled to a pre-column (5 × 2 mm, 1.8 μm) thermostated at 25 °C. The flow rate was set at 0.5 mL/min while the volume of injection was 5 μL. Mobile phases used were as follows: water (A) and methanol (B), both acidified with formic acid (0.1%) and ammonium formate (5 mM). As for the gradient, B started with 10% for 0.5 min and afterwards increased up to 2.5 min to 80%, followed by an increase to 100% during 3 min, and a decrease to 10% in 2 min. Lastly, the column was balanced at 10% for 1.5 min before starting the next injection. The total run-time of the method was 9 min.

Mass spectrometry analysis was performed by using an electrospray ionization interface (ESI) working in positive ion mode. The acquisition methods were set to obtain the full scan and fragmentation spectrums with all independent ions (AIFs) modes. Scan parameters for full scan mode were the following: mass resolving power of 35,000 FWHM, scan range 80–1200 *m/z*, automatic gain control of 1×10^6, maximum time of injection of 200ms and a scan rate of 3 scan/s, sheath gas flow rate: 40 arbitrary unit (a.u.), auxiliary gas flow rate: 10 a. u., spray voltage of 2.8 kV, heater temperature: 305 °C, capillary temperature: 310 °C, capillary voltage: 50 V and tube lens voltage: 110 V. Scan parameters for AIF mode were the following: 17,500 FWHM mass resolving power, 80–1200 *m/z* scan range, 1×10^5 automatic gain control, 200ms maximum time of injection, 3 scan/s scan speed with 0.10s of scan time, isolation window up to 5 *m/z*, and retention time at 30s. Processing of data obtained was performed with Xcalibur software, v.3.1.66.10 (Xcalibur, Thermo Fisher Scientific, Waltham, MA, USA).

2.5. Validation parameters

Method was validated for the analysis of mycotoxins residues in liver samples. All the results were processed according to European standards EC 2021/808, EC 2002/657, EC 401/2006 (Commission Regulation, 2006), SANTE criteria (SANTE/12682/2021). Several parameters for validation were tested following the guidelines mentioned. Among them, sensitivity/linearity, matrix effect, specificity, intra-day and inter-day repeatability (RSDr) and reproducibility (RSD_R) for spike tested, LOQ, robustness, ion ratio, retention time of the analytes. The latter was compared with standards, with a tolerance of ±2.5%. For quality assurance and quality control, both reagent and procedural blanks were included, as well as triplicate samples, and a matrix-matched calibration in each batch of samples. Moreover, three spiking levels for each stock solution were used. Mean recovery values, as a measure of trueness or bias, were evaluated within the range of 70–120% with relative repeatability RSDr ≤20%.

2.6. Statistical analysis

Data regarding rats' parameters (weight, weight gain, feed intake, toxin intake) were calculated weekly during 28 days and expressed as mean ± SD among male and female individuals of each group. Statistical analysis of the results was performed by using Student t-test for paired samples, considering $p\leq0.05$ as statistically significant. Regarding mycotoxin residues in liver, results were presented as mean ± RSD. InfoStat 2008 was used to perform the statistical analysis of the data, setting a $p\leq0.05$ as statistically significant. Graphical representations of the data were created with GraphPad Prism software version 8.0.0 (San Diego, California, USA).

3. Results and discussion

3.1. Body weight and liver variations in rats

The BW of each rat (male and female) was gauged every week from the beginning of the experiment to mark the effect of exposure progression. Weight variations from week 1–4 are illustrated in Fig. 2. Generally, weights were higher in male than in female, ranging from 192.60 to 377.26±37.66g and 130.40–242.54±26.66g, respectively. Considering different groups individually, statistically significant decreases were observed in presence of AFB1 and combined exposure of mycotoxins (AFB1+OTA), especially in males (Fig. 2A and 2B). On the other hand, in presence of functional ingredients, weights gradually increased in nearly all cases (Fig. 2C, 2D, 2E, 2F), reporting significant effect on BW gain. Similarly, other authors observed a significant decrease in weight gain of rats to AFB1 which was clearly declined by the addition of probiotic supplementation (Sahin et al., 2022). Likewise, caffeic acid exerted an ameliorative action on rats BW during 28 days of AFB1 exposure (Owumi et al., 2022). Single weight values of individuals and weight gain are reported in supplementary material 3.

Relative liver weight (LW) was calculated on rats BW for both sexes. When comparing individuals of the same batch (solely mycotoxins, FW or FW+P), no significant diminishments were evidenced in mycotoxin groups ($p>0.05$). However, a significant reduction ($p<0.001$) was observed in females for OTA+FW, while in males, an increase was noted ($p<0.01$) (Fig. S1). According to that, another toxicological study on OTA reported no significant changes in BW and LW of Wistar rats after administration of similar concentrations (289 μg/kg) for 90 days (Gagliano et al., 2006). Furthermore, the same scenario occurred in rats fed AFB1 at four different doses (4, 10, 25, 50 μg/kg) during 60 days and 150 μg/kg for 2 weeks (Qian et al., 2016; Wei et al., 2014). As for rats fed with both ingredients, males were affected by AFB1 and OTA exposure, but not female. Additionally, all groups exposed to combined AFB1 presented statistically significant increase ($p<0.05$) compared to rats to only AFB1. Similarly, the addition of curcumin and lycopene alleviated AFB1 weight loss after 30 days exposure (Wang et al., 2022; Xu et al., 2017).

3.2. Feed intake and toxin intake

The average of feed intake in the experimental conditions is reported in Table S2. Groups free from functional ingredients, both female and male ingested lower quantities comparing to the ones fed with FW and P individually or combined. Generally, female rats consumed smaller amounts in all groups than male (almost all cases up to 10 g/day), especially groups fed with AFB1, ranging from 5.7 to 9.4 g/day. Notably, groups supplemented with FW ingested reduced amounts of feed in presence of mycotoxins compared to the control almost every week. Moreover, in some cases with the mixture of mycotoxins, quantities were lower than OTA alone. Regarding mycotoxin PDI (Eq. (1)), the overall consumption of AFB1 was lower than OTA (Table S3), probably due to the differences in feed composition. As for mortality, one case was recorded during the third week of exposure belonging to FW+AFB1+OTA group. However, causes were not related with the exposure according to veterinary report. No clinical signs attributable to toxic effect were observed.

3.3. Optimization of the UHPLC-Q-Orbitrap HRMS parameters

The optimization of chromatographic conditions was initiated via

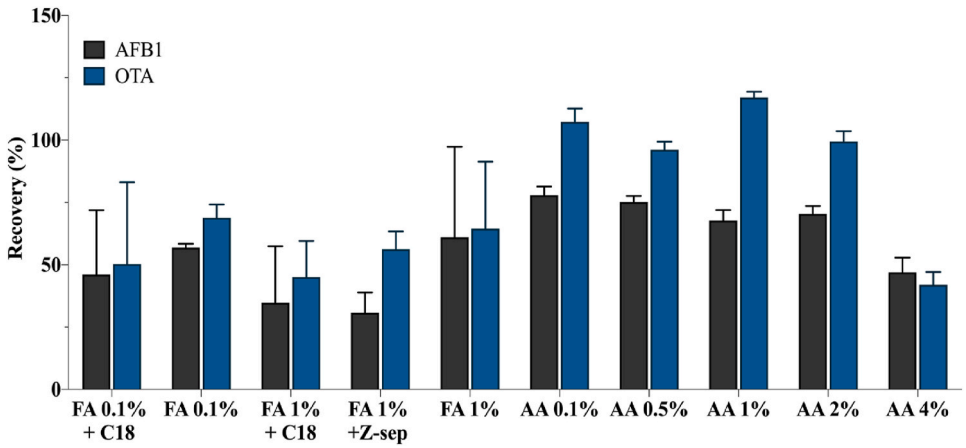

Fig. 3. Recovery of mycotoxins from liver. RE values were obtained by applying the extraction based on acetonitrile (ACN) acidified with formic acid (FA) at different concentrations (0.1, 1%), with or without clean-up steps (C18 or Z-Sep), ACN acidified with acetic acid (AA) at different concentrations (0.1, 0.5, 1, 2, 4%) without clean-up step for AFB1 (dark blue columns) and OTA (light blue columns). Bars represent standard deviations (SD) among the replicates.

direct infusion of AFB1 and OTA standards into the Q-Orbitrap system, which were diluted at 1 μg/mL with a flow rate 10 μL/min. Regarding mobile phases, water was chosen as the polar phase and methanol as the organic phase, due to its higher sensitivity in mycotoxins detection compared to ACN (De Colli et al., 2020). Moreover, formic acid (0.1%) and ammonium formate (5 mM) were added to the solutions in order to improve the ionization efficiency and achieve an optimal chromatographic separation. The most intense signals were selected for both mycotoxins, as well as the stable adducts. In order to perform the precursor ions fragmentation, different collision energies were implemented: 36eV for AFB1 and 16eV for OTA. UHPLC-HRMS parameters for AFB1 and OTA determination are shown in Table S4.

3.4. Optimization of sample preparation

The extraction of mycotoxins from biological solid matrices requires the optimization of very selective steps owing to the complexity of components that might interfere in analyte purification and recovery (Escrivá et al., 2017). Nowadays, the most employed procedure is based on QuEChERS (quick, easy, cheap, effective, rugged, and safe) methodology, which consists of an extraction step via salting-out for the separation of aqueous-organic solvent after mycotoxin migration and a further clean-up process to remove interfering substances (Perestrelo et al., 2019). In the present research, several extraction parameters were assessed in order to select the highest performing parameters for AFB1 and OTA determination (Fig. 3). Among them, the optimization of extracting solvent and clean-up step. All the procedures were carried out in triplicate using three different fortification levels at 50, 25, 5 ng/mL.

3.4.1. Optimization of extracting solvent

The experiments for mycotoxins extraction in liver samples were based on a liquid-liquid extraction assisted by salting out method, using ACN as extracting solvent. This solvent has been widely used for analytical extraction of these toxins in several tissues, being a water-miscible organic substance (Arroyo-Manzanares et al., 2021). Nevertheless, its acidification with formic acid (FA) or acetic acid (AA) markedly improved the extraction procedure by preventing the linkages with other constituents of the sample, such as proteins (Cao et al., 2018). In this study, different levels of acidification have been tested. Firstly, ACN was mixed with FA at concentrations of 0.1% and 1%, reporting recoveries ranging from 30 to 61% for AFB1 and 41–64% for OTA. In addition, notable differences between spiking levels stood out, reporting SD up to ±36% for AFB and ±34 for OTA. Thus, the recovery in these conditions was unsatisfactory for both mycotoxins. Subsequently, AA was employed at several concentrations (0.1, 0.5, 1, 2 and 4%) without clean-up sorbents, showing most effective results. Concentrations of 1% showed the highest recoveries for OTA (118%) and 0.1% for AFB1 (80%). Among all, 0.1% presented the most suitable recoveries for the co-occurrence of AFB1 and OTA (80% and 108%, respectively).

3.4.2. Optimization of clean up

As for salting-out, the combination of magnesium sulfate ($MgSO_4$) and sodium chloride (NaCl) have shown to be the most widely used in QuEChERS extraction (Socas-Rodríguez et al., 2017). According to literature, 150 mg $MgSO_4$ and 50 mg NaCl were employed in this study as salting-out sorbents, reporting higher sample cleanliness. Given the complexity of the matrix, a purification step was initially included to remove the compounds which may interfere with mycotoxins detection. Among the most widely used sorbents, C18 is best suited to remove fatty acid and non-polar components (Mateus et al., 2021). In this study, C18 was tested (50-25 mg) to increase the effectiveness of ACN acidified with AA at 0.1%. In both cases, recovery values were lower than procedures that included only salting-out step. Likewise, the clean-up efficiency was investigated using Z-Sep sorbent (25 mg), which has improved AFB1 and OTA detection in various studies (Perestrelo et al., 2019). However, in this case did not show satisfactory findings, resulting in a signal suppression of mycotoxins.

3.5. Method validation

The UHPLC-HRMS parameters of mycotoxins included in this study are reported in Table S5. Chromatographic spectra obtained in full scan and fragmentation mode of each analytical standard is shown in Figs. S2–S3. Calibration curves were built at 8 levels of concentration within a linear range from 0.01 to 200 ng/mL, reporting regression coefficients (r^2) above 0.9996. Matrix effect (SSE) was calculated comparing calibration curves constructed in solvent (external curve) and in blank matrix (internal curve), showing the absence of interferences with the matrix (>98%). Therefore, external curves prepared in the matrix were selected for the quantification. LOQ were 0.195 for AFB1 and 3.120 for OTA. Blank samples were then spiked in order to calculate recovery values (%) of both targeted mycotoxins, reporting ranges of 72–76% for AFB1 and 77–87% for OTA. Moreover, according to EC 2021/808, intra-day and inter-day relative standard deviations values (RSDr, RSD_R) were below 20% for both studied compounds. The proposed methodology has proved to be very accurate in AFB1 and OTA determination in liver samples.

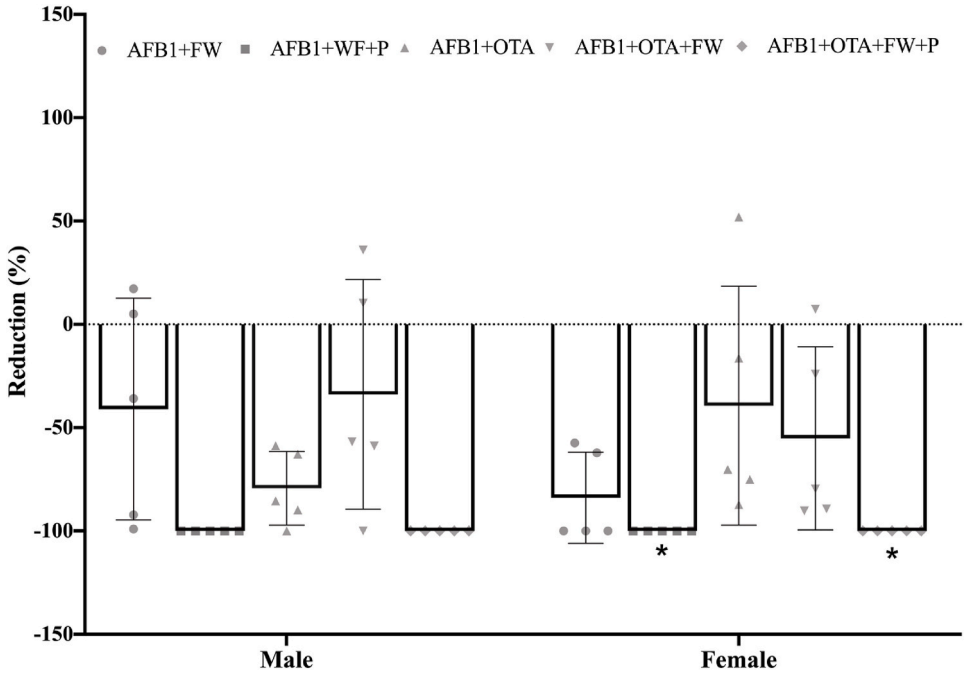

Fig. 4. Reduction (%) of AFB1 on the residues in liver tissues normalized with its daily intake in male and female, compared with single mycotoxin exposure. Results refers to mean values of male and female and expressed in percentage scale compared to AFB1 group. *p ≤ 0.05; The average was calculated considering all individuals of the same group and the same sex. Error bars represent the standard deviations (SD). Each symbol represents a condition of exposure. FW: Fermented whey, P: Pumpkin, AFB1: Aflatoxin B1, OTA: Ochratoxin A.

3.6. Mycotoxins occurrence in rat liver samples

The methodology validated formerly was applied to the selected liver samples (n=120), highlighting the presence of both mycotoxins in almost the entirety of individuals. The total of mycotoxins found in the sample set analyzed are shown in Table S6. Regarding AFB1, very low quantities of this toxin have been detected compared to the initial ingestion rates (Table S3). According to this, despite the liver is the main target organ of the toxin, it is absorbed from food through the intestinal tract, bio transformed to metabolites and mainly excreted via urinary route (Thieu & Pettersson, 2009). However, its prolonged exposure causes its accumulation in liver by inducing hepatotoxicity and the impairment of hepatocytes cycle (Xu et al., 2021). In the groups of rats that ingested only AFB1, the totality of experimental male presented mycotoxin residues ranging from 0.882 to 5.790 μg/kg which were lower than in female (3.886–15.560 μg/kg). Likewise, when adding FW to the diet, residues were upper in female, especially in the individual exposure (6.093±4.525 μg/kg), although only the 40% of the samples were positive to the toxin. These findings are consistent with previous studies suggesting that sex hormones may influence the oxidative stress response and detoxification processes, potentially making females more susceptible to hepatotoxic effects (Allegra et al., 2023; Xu et al., 2022). On the contrary, in presence of both functional ingredients, no mycotoxin was detected. Moving to OTA, significant toxin residues were observed after 28 days assay, especially when administered alone (211.750±51.181 in male, 238.984±75.187 μg/kg in female), presenting a higher percentage of positive samples than AFB1 (80–100%). In fact, considering OTA mechanism of action, it is swiftly absorbed in the organism owing to its high binding affinity to plasma proteins and then distributed in hepatocytes (Tao et al., 2018). Nonetheless, quantities were lower after FW and pumpkin supplement, being the combined exposure with AFB1+OTA+FW+P once again the most effective (74.506±17.087 μg/kg; 72.218±44.458 μg/kg).

3.7. Mycotoxins reduction

The current study focused on the assessment of AFB1 and OTA behavior under different experimental conditions, simulating their natural proliferation in the production process. In fact, the mycotoxin-synthesizing fungi were used to naturally contaminate the flours employed in the preparation of the feed, which was then administered to the animals. For the experiment, rats were employed as experimental model, serving as an excellent specimen for assessing the impacts of human exposures to mycotoxins, as it closely mirrors the human pathways of mycotoxins metabolism (Furian et al., 2022). Additionally, particular attention has been paid on the possibility of using natural functional ingredients (FW and P) rich in bioactive compounds in foodstuff as a mitigation strategy for reducing mycotoxins concentration once absorbed at hepatic level. The efficiency of bioactive ingredients likely relies on their ability to activate cytochrome P450 enzymes, which play a crucial role in the activation of both phase I and phase II detoxification pathways of xenobiotics. Indeed, a recent study has demonstrated the effectiveness of FW and P in activating this metabolic pathway for AFB1 and OTA detoxification in liver (Trombetti et al., 2025). This activation enhances the ability of the body to metabolize and eliminate harmful xenobiotics, suggesting that these bioactive ingredients could contribute to improved detoxification processes and overall health maintenance.

For this investigation, mycotoxin residues in the liver were normalized to their corresponding daily intake ratio to determine the percentage reduction in relation to individual mycotoxin exposure. Remarkably, these functional ingredients reduced AFB1 concentration until 100 % in almost all conditions studied (Fig. 4), being statistically significant in female with FW+P ($p<0.05$). This is consistent with several studies reporting the detoxification ability of microorganisms such as LAB through the absorption and degradation of AFB1 (Wang, Huang, et al., 2022). On the other hand, the results are more significant in females than in males, likely due to the ability of testosterone to further activate the metabolic mechanisms of AFB1 (Huang et al., 2024). Nevertheless, the mixture of both mycotoxins with or without FW have

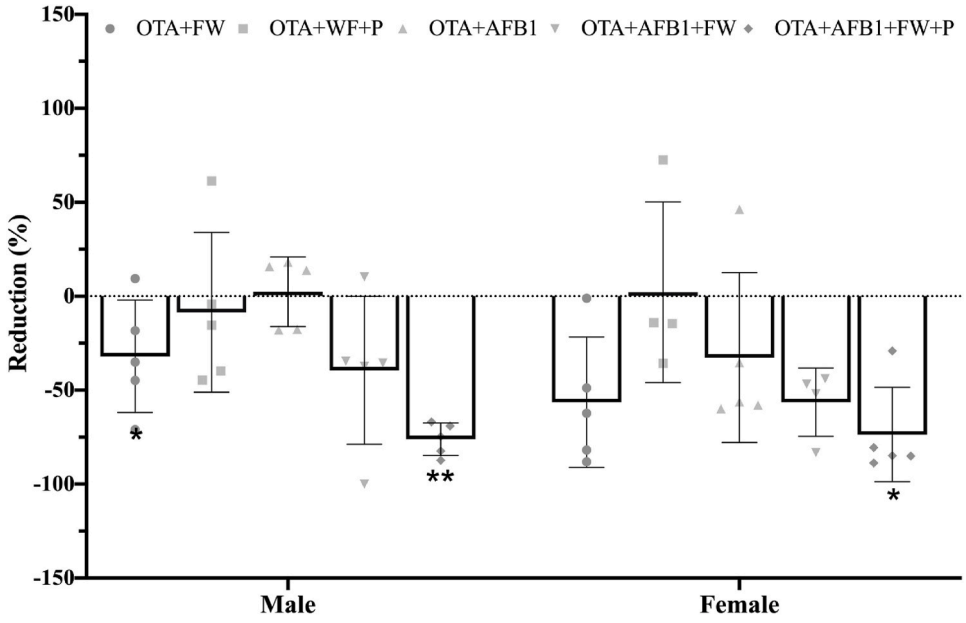

Fig. 5. Reduction (%) of OTA residues in liver tissues normalized with individual daily intake in male and female, compared with single mycotoxin exposure. Results refers to average values of male and female and expressed in percentage scale compared to OTA group. *$p \leq 0.05$; **$p \leq 0.01$; The average was calculated considering all individuals of the same group and the same sex. Error bars represent the standard deviations (SD). Each symbol represents a condition of exposure. FW: Fermented whey, P: Pumpkin, AFB1: Aflatoxin B1, OTA: Ochratoxin A.

shown less reduction, maintaining a lower trend in female (54-43%) compared to male (79-33%). This is in line with similar findings in animals fed with naturally contaminated feed with AFB1 and OTA, in which residual concentrations levels in liver were significantly higher in individual exposure than in combination (Hassan et al., 2012).

Analyzing the activity of OTA (Fig. 5), the mixture of the toxins blended with FW and P resulted the most effective ($p<0.01$ male, $p<0.05$ female) in its diminution (78%), followed by OTA+FW (40% male, 60% female). Moreover, a considerable reduction was observed in female in presence of both toxins (54%) and with FW (38%). Lower values were encountered in males for the same conditions (both mycotoxins 24%, FW 33%). In the case of OTA, the bio-detoxification mechanisms are likely attributed to the binding of this molecule to the cell wall of LAB, forming an OTA-cell complex that renders the original activity of OTA ineffective, thus detoxifying it (Mwabulili et al., 2023). The presence of highly antioxidant molecules in pumpkin enhances these mechanisms. Overall, as can be seen from the results obtained, it is important to highlight the difference in reduction between AFB1 and OTA. In fact, in some cases, AFB1 showed a total residual elimination in the liver. In contrast, OTA showed a significantly lower reduction.

4. Conclusion

The present work has validated an efficient methodology to simultaneously quantify AFB1 and OTA residues in rat liver through a UHPLC-Q-Orbitrap HRMS determination. The analytical procedure was successfully applied to 119 individuals to mycotoxins and functional ingredients (FW, P), allowing the quantification of both toxins in roughly the totality of specimens. Moreover, the capability of FW and P in minimizing toxins accumulation was calculated depending on the corresponding daily intake of AFB1 and OTA. Interestingly, in feed enriched with FW+P was observed a 100% reduction of the initial concentration (feed contaminated with AFB1), either in male than female. However, with only FW or in combination with OTA reductions were smaller. Regarding OTA, the reduction maintained the same trend being higher in presence of both ingredients but in this case, the co-occurrence with AFB1promoted the lessening, especially in female. Overall, the mixture of FW and P in feed formulation has proven to be an important strategy for reducing mycotoxins bioaccumulation in liver, which can be easily introduced in food industry, preserving the health of consumers.

CRediT authorship contribution statement

Alessandra Cimbalo: Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Juliane Lima da Silva:** Validation, Methodology, Formal analysis, Conceptualization. **Luana Izzo:** Validation, Methodology, Investigation. **Alberto Ritieni:** Visualization, Project administration, Conceptualization. **Lara Manyes:** Writing – review & editing, Visualization, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2025.106565.

Data availability

Data will be made available on request.

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